Adenovirus – a blueprint for gene delivery

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Adenovirus - A blueprint for gene delivery

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Abstract

A central quest in gene therapy and vaccination is to achieve effective and long-lasting gene expression at minimal dosage. Adenovirus vectors are widely used therapeutics and safely deliver genes into many cell types. Adenoviruses evolved to use elaborate trafficking and particle deconstruction processes, and efficient gene expression and progeny formation. Here, we discuss recent insights into how human adenoviruses deliver their double-stranded DNA genome into cell nuclei, and effect lytic cell killing, non-lytic persistent infection or vector gene expression. The mechanisms underlying adenovirus entry, uncoating, nuclear transport and gene expression provide a blueprint for the emerging field of synthetic virology, where artificial virus-like particles are evolved to deliver therapeutic payload into human cells without viral proteins and genomes.

Short title: Entry and early gene expression of adenovirus

Keywords:

Virus entry; uncoating; receptor; endocytosis; membrane penetration; cytoplasmic transport; nuclear import; E3 ubiquitin ligase; E1A gene expression; virus-like particle; laboratory virus evolution; nucleic acid packaging; anti-virus immunity;

Highlights for discovery

-Stability and built-in weaknesses are emergent properties of adenovirus particles and key for gene delivery

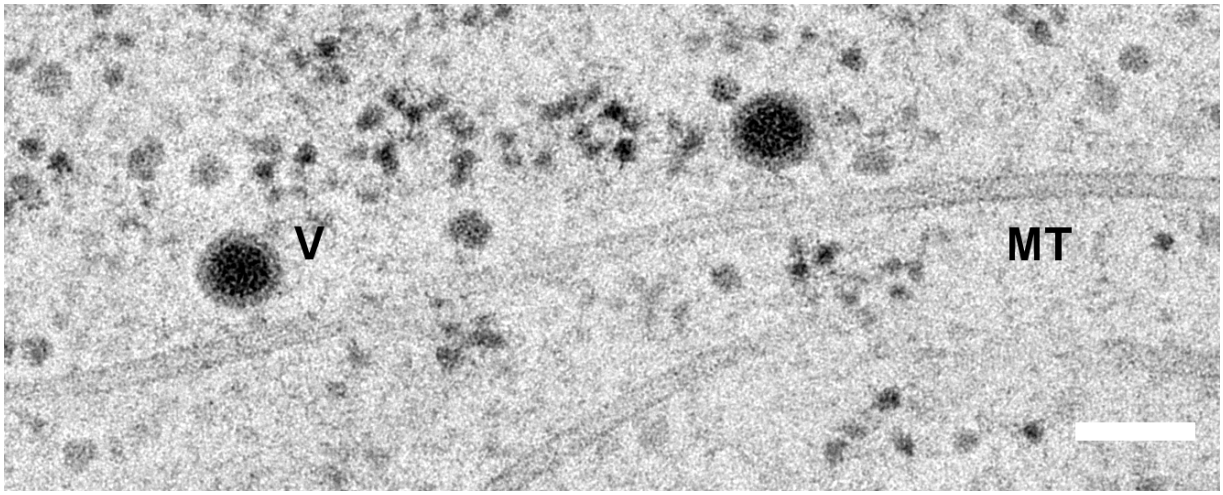
-Entry is a gain of function process where the virion uncoats in a stepwise manner and adapts to the subcellular environment

-The ubiquitin ligase Mib1 triggers the release of the DNA genome from the capsid at the nuclear pore complex

-Adenovirus gene expression exhibits cell-to-cell and genome-to-genome variability with lytic or persistent outcomes

Graphical abstract

Human cervical epithelial HeLa cells grown on sapphire coverslips were infected with HAdV-C5 at a high multiplicity in presence of leptomycin B for 5 h, high pressure frozen using liquid nitrogen at 2100 bar, and freeze-substituted. Thin sections of 70 nm thickness were imaged using transmission electron microscopy at the Center for Microscopy and Image Analysis (ZMB) of the University of Zurich. (V) Virus particle, (MT) microtubule. Pixel size 0.8 nm, scale bar 100 nm.



Introduction

Viruses are highly prevalent and diverse, yet specialized and typically well adapted to their hosts. They propagate and spread within and between organisms, package a nucleic acid genome into a protein capsid and unpack the same genome upon entry into a naive host cell. Viruses evolved intricate assembly and disassembly processes. For some viruses, such as bacteriophages, these processes are understood at the level of molecules, biophysical features and nucleotide precision genetics [for overviews, see 1,2,3]. Challenges remain, however, to better understand the complex processes occurring in virus infection of eucaryotic cells.

~~The~~ Studies of human adenovirus (HAdV) ~~interactions with cells~~ have a long track record, with seminal contributions from molecular and cell biology, virology, immunology, and more recently gene therapy and vaccinology [for recent reviews, see 4,5-8]. HAdVs are grouped into seven species (A-G) and comprise more than 100 types, as indicated by serology and genome sequencing [reviewed in 9,10]. Their number currently exceeds that of known animal adenoviruses. HAdVs infect the respiratory and the gastrointestinal organs, the kidneys and the eyes, and are life-threatening in T cell suppressed individuals [reviewed in 11]. A typical HAdV genome has about 36 kbp, and encodes 30-40 distinct proteins in early (E) or late (L) genes [12], not counting potential proteins from more than 11’000 alternatively spliced mRNAs [13]. The AdV genome replicates in the nucleus and leads to the formation of discrete zones of virion assembly in this organelle.

The assembly of AdV particles

Adenoviruses have high structural similarity to bacteriophages [reviewed in 3]. Their assembly is an elaborate process depending on the large abundance of capsomers, genomes, and assembly factors. AdV capsomers are put together in specialized domains of the nucleus, so called replication zones. Although AdVs encode a putative ATP-binding protein IVa2 implicated in viral DNA packaging, and give rise to empty capsids lacking viral DNA [14-16], they do not use empty capsids as an intermediate in the formation of infectious virions, unlike herpes viruses, which also replicate a linear double-stranded DNA genome in the nucleus. Empty HAdV capsids are stable, and are

most likely a defective assembly product, as they do not mature into infectious particles [17,18]. HAdV capsids package viral DNA both in presence and absence of the major viral DNA condensing protein VII [19,20]. Protein VII firmly associates with newly synthesized viral DNA in the nucleus, is packaged into progeny virions, and remains associated with the viral genome until delivery into the nucleus in a new round of infection [21,22]. It is unlikely that a DNA packaging machinery pumps two very different substrates, viral DNA with or without protein VII through a hypothetical portal complex into preformed empty capsids. It is more likely that packaging of the HAdV genome occurs by coordinated interactions of different viral proteins, including IIIa, L1 52/55K, L4 33K, L4 22K and IVa2.

The organisation of the adenoviral DNA in the capsid has remained elusive, although several mature capsids were resolved at near atomic details, including the respiratory C5, the ocular D26 and the enteric F41 [23-26]. Their icosahedral structures reveal a tight interconnection of the major capsid protein hexon and various minor proteins [25,27]. The capsid and protrusions from the vertices give rise to emergent properties of the virion, including stability, cell receptor binding but also physico-chemical weaknesses [reviewed in 28,29]. For example, the penton base proteins at the vertices readily detach upon application of mechanical force to single isolated virions by atomic force microscopy ~~AFM~~, or by receptor engagement on the plasma membrane of host cells [30-32]. The detachment of the fibers from the incoming virion [33], which binds with high affinity to its receptor [34], is important to release the particle to the cytosol, and facilitate cytoplasmic trafficking to the nucleus. The release of the penton base is thought to open up the particles to release the membrane lytic protein VI [for review, see 35].

Adenovirus attachment to cells

The fiber knob is a major binding determinant for most HAdVs on cells and tissue. Its tertiary structure is conserved across the seven different HAdV species, but variable in amino acid composition, particularly in the hypervariable loops. In most cases, the knob attaches to the coxsackievirus AdV receptor (CAR), CD46, desmoglein (DSG) 2, or sialic acid [reviewed in 36]. These receptors vary in abundance on target cells, as well as affinity for the particular fiber knobs. Since the fiber knobs occur in as many as 12

homotrimers per virion, they overcome low affinity binding to receptors by avidity engagement, as shown initially for the HAdV-B3 fiber knob binding to the ubiquitous membrane cofactor CD46 [37]. Fiber knobs may also bind to not just one receptor, but multiple ones, for example as suggested for the vaccine vector HAdV-D26, which binds CAR and sialic acid with low affinity [38,39], and engages $\alpha_V\beta_3$ integrins through penton base [40].

Increasing evidence demonstrates that besides fiber knob and penton base, hexon is an important mediator of HAdV binding to particular cell types. For example, the hexon hypervariable region HVR1 attaches HAdV-B35, HAdV-D26 and HAdV-C5 to the scavenger receptor SR-A6 (MARCO) of alveolar-like macrophages, leading to macrophage transduction and cytokine production, as shown in knock-out alveolar-like macrophages and mouse models [41,42]. The D species comprises the largest number of HAdV types among all the seven species. Some D types, such as the vaccine vector HAdV-D26 have either low or high seroprevalence depending on the region of the globe, whereas others, such as HAdV-C5 are endemic in many countries [reviewed in 7,43,44]. Vectors derived from HAdV-D26 but also HAdV-C5 are used as vaccines against COVID-19 [45], and HAdV-B35 based vectors are employed in hematopoietic stem cell transductions [46]. Hexon of HAdV-D56 particles binds to CD46, and a large excess of soluble CD46 inhibited the infection of epithelial cancer cells with HAdV-D26 [47]. These data suggest that besides CAR and sialic acid, SR-A6 and CD46 may be involved in infections with species D HAdVs.

Endocytosis, membrane penetration and cytoplasmic transport

Infection and transduction require the uncoating and delivery of AdV genomes into the nucleus. The AdV entry and uncoating program is best described for species C HAdV types 2 and 5, and in principle applies to viruses of other species (Fig. 1). Mechanical forces acting on the HAdV-C particles expose the internal membrane lytic protein VI [reviewed in 29,35,48,49]. This is a key step and occurs when the virion is in contact with the appropriate receptors, such as coxsackievirus adenovirus receptor (CAR) and α_v integrins. Within the virion, the exposure and the release of protein VI is controlled

by a tunable mechanism, where the DNA-condensing protein VII competes with VI for binding to hexon, and poises the particle for exposing protein VI [50]. In absence of VII, VI cannot be exposed and virions fail to penetrate to the cytosol. Following dynamin-mediated endocytosis, the exposed protein VI preferentially binds to ceramide lipid-rich membrane domains, and ruptures the limiting endosomal membrane. The partly dismantled capsid then uses dynein- and kinesin-mediated transport on microtubules to the nucleus, binds and uncoats at the nuclear pore complex (NPC), and imports a double-stranded linear DNA genome in complex with viral proteins into the nucleus [reviewed in 29]. Likewise, the ocular HAdV-D37 enters human corneal epithelial cells by a clathrin-mediated pathway independent of low pH, but unlike HAdV-C5 involves caveolae-signalling, lysosome-associated membrane protein 1 and the kinase PAK1, the latter akin to HAdV-B3, a hallmark of late-penetration [51,52]. Unlike the early penetrator HAdV-C5, the entry of HAdV-D37 into keratinocytes was enhanced by siRNA-mediated knock-down of dynamin-2, and inhibited by overexpression of dynamin-2, possibly involving alterations of microtubule dynamics [53]. The latter could relate to virion trafficking from the microtubules to the NPC, which requires a nuclear factor for virion detachment from microtubules proximal to the nucleus [54].

Ubiquitination controlled AdV DNA uncoating at the NPC

HAdV particles dock to the cytosolic side of the NPC, disassemble and deliver their genome through the NPC into the nucleus [reviewed in 55]. A genome-wide RNA interference screen with HAdV-C2-ΔE3B-CMV-GFP revealed the RING-type E3 ubiquitin ligase Mind bomb 1 (Mib1) as a proviral host factor for HAdV infections [56]. Mib1 is involved in embryogenesis and anti-viral RNA innate immunity. Its depletion strongly blocked HAdV-C2/5, A31, B3 and D8 infections, arrested incoming particles and their genomes at NPCs and inhibited viral DNA nuclear import. Induced expression of full-length but not ligase-defective Mib1 in knockout cells triggered vDNA uncoating from NPC-tethered virions, nuclear import and viral infection. These results were phenocopied by a recent gene-trap screen using a replication-defective HAdV-C5 vector in haploid cells [57]. In both studies (Bauer et al. and Sarbanes et al.), HAdV infection required the ubiquitination activity of Mib1, suggesting that Mib1 signals to dissociate or degrade cellular or viral factor(s), which block uncoating. One class of

cellular factors blocking the attachment of HAdV-C2 with NPCs in vitro are ribonucleoprotein (RNP) particles [58]. Interestingly, a proteomic screen of HAdV infected cells identified RNP proteins to be ubiquitinated in proximity to Mib1 [57]. The results suggest a scenario where deubiquitinated RNPs shield the HAdV from uncoating, whereas ubiquitinated RNPs are permissive for HAdV uncoating.

In addition to potential host factors, Mib1 targets the virion protein V [59]. Protein V constitutes nearly 30% of the protein mass of the viral DNA-core, while 70% is from protein VII, protein X and the terminal protein. Protein V links the core with the capsid wall via protein VI. It dissociates in two steps from the incoming particle. In the early phase of entry about 65% of V are released, and during DNA uncoating at the NPC the remaining is released [21]. HAdV-C5 particles lacking protein V poorly infect normal cells, unlike wild-type virus, but readily infect Mib1-knockout cells [59]. In normal cells, the V-minus HAdV-C5 releases the genome before reaching the NPC, which triggers the production of chemokines and interferon in response to the DNA sensor cGAS. Likewise, HAdV-C5 particles containing non-ubiquitinable protein V (all lysine residues mutated to arginine) showed reduced nuclear import and enhanced levels of cytosolic viral DNA. The data show that protein V serves as a linchpin in the virion suppressing the exposure of pathogen-associated molecular patterns. Mib1 triggers the quantitative release of protein V from the virion, and thereby primes the virion for kinesin-mediated rupture, followed by nuclear import of the viral DNA and infection [60].

In summary, the HAdV entry and uncoating processes have features best described as a sequence of discrete steps, but without evidence for an empty capsid intermediate at the NPC. This is distinct from herpes viruses, which open up a single vertex upon arrival at the NPC, release the double stranded DNA and leave an empty capsid in the cytoplasm [reviewed in 55].

Early gene expression from incoming viral DNA

A central quest in viral gene therapy and vaccination is the effective and long lasting expression of exogenous genes of interest at minimal vector dosage. This is difficult to achieve because the vector of choice may transduce insufficiently due to a paucity of cell receptor expression [61,62], limited uncoating of its genome, aberrant intracellular

trafficking, or innate and adaptive immunity precluding vector gene expression. Genomes of DNA viruses, such as AdVs, are transcribed by host RNA polymerases (Pol), and suppressed by innate immune responses, conditions that lead to virus persistence albeit without integration into the host genome [for review, see 11].

For example, the immediate early protein E1A drives AdV infection by controlling the subviral and numerous cellular promoters, except those controlling the expression of virus-associated (VA) RNAs, which require RNA Pol III [63]. Together with the anti-apoptotic E1B-19K and the multifunctional 55K proteins, E1A controls pathways for cell proliferation, differentiation and homeostasis. The latter involves the endoplasmic reticulum unfolded protein response (UPRER), where it engages the transmembrane sensor Ire1a in a transcriptional feed-forward loop [reviewed in 64]. Ire1a has a kinase and nuclease activity. The nuclease is triggered by the viral E3-19K glycoprotein in the ER lumen, and catalyzes the splicing of the mRNA encoding X-box binding protein (XBP) 1, yielding active transcription factor XBP1s, which binds to the E1A promoter and enhances E1A transcription [65]. A loop of five components (E1A, E3-19K, Ire1a, XBP1s and the E1A-promoter) stimulates lytic infection outcome, and viral persistence in presence of the anti-viral cytokine interferon [65,66]. The maintenance of E1A expression is important for long lasting viral gene expression, since E1A suppresses the expression of interferon-stimulated genes by inhibiting, for example, the E3 ubiquitin ligase hBre1 [67,68].

But the picture is more complex, as indicated by the heterogeneity of cell responses to infection. Recent single-cell, single-genome studies with HAdV-C5 revealed high cell-to-cell variability of E1A expression, ranging from nuclei with as many as 25 viral genomes and no E1A expression to others with as few as one or two genomes and more than 30 E1A transcripts [69]. This heterogeneity is observed at early time points post infection, but later almost all infected A549 cells become loaded with E1A mRNAs, before these transcripts disappear at the later stages of infection. Despite this heterogeneity, there is a clear correlation between the number of viral genomes and E1A transcripts, and this correlation is increased in G1 cells, suggesting that the cell cycle state contributes to the heterogeneity of infection, albeit mildly.

Viral chromatin remodelling is an important process controlling AdV gene expression. The incoming viral genome is condensed with the DNA binding protein VII, which shields repulsive DNA-DNA interactions, and reduces the internal pressure in the virion [20]. Protein VII also condenses the viral DNA in the penton-low cytoplasmic capsids trafficking in endosomes and the cytosol [70]. Upon disruption of the capsid at the NPC, protein VII accompanies the viral DNA into the nucleus, but its role in the nucleus has been debated. Some evidence suggests that protein VII supports early viral transcription [71,72], while other data argue that its removal from the viral DNA is essential for transcription and replication [73,74]. Positional heterogeneity of protein VII on the viral DNA may account for some of the observed variability in E1A expression, and viral replication. Regardless, limiting dilution and live cell plaque formation experiments have recently shown that the outcome of AdV infection is three-fold: aberrant (without the spreading of progeny to neighboring cells), non-lytic (small symmetrical plaques) and lytic (large smeary plaques) [75]. Microscopy machine-learning revealed a distinction between lytic and non-lytic infected cells, namely the intranuclear pressure increased in lytic infected cells compared to non-lytic cells [76]. Future investigations will continue to take advantage of predictive imaging from machine-learning and explore the infection variability, which has been a long-standing challenge to both biologists and virologists.

Synthetic viruses

One approach to address viral mechanisms is synthetic virology. This field may help to understand fundamental problems, for example along the famous note of Richard Feynman ‘What I cannot create I do not understand’. Synthetic virology engineers virus-like particles, ideally to deliver cargo on demand to any cell of interest in the body. Such virus-like particles are not found in nature, and mimic features of viruses, including cubic, icosahedral, or helical assemblies, scaffolds, glues, and cores of nucleic acids and proteins [reviewed in 77]. Arguably, a foundation for synthetic virology was laid with the chemical synthesis of poliovirus [78], and extended for example by the reversible assembly of virus-like protein cages under the control of metal coordination at the protein interfaces [79]. More recently, laboratory evolution of bacterial enzymes, such as the *Aquifex aerolicus* lumazine synthase (AaLS) involved in riboflavin synthesis gave

rise to virus-like capsids that package RNA [80,81]. The synthetic evolution process has been expanded by complementary adaptations between cargo and container to T=4 particles, which selectively package RNA [82]. This was achieved by including the lambda N peptide at the amino terminus of AaLS and flanking the capsid-encoding RNA with a stem loop structure recognized by lambda N. So far, however, the capsids have remained unresponsive to uncoating cues and failed to release RNA or penetrate into the cytosol of target cells. In analogy to natural viruses, this suggests that further components are required to achieve proper cargo delivery to target cells. It will be essential to build in a tunable endosomal escape machinery, akin to the host factor controlled release of the membrane lytic protein VI of HAdV [83,84]. Intriguingly, the membrane lytic domain of protein VI genetically fused to the vault poly(ADP-ribose) polymerase interaction domain was active in mediating the co-delivery of a soluble ribotoxin or plasmid DNA into murine macrophages [85].

The limited cargo carrier capacity of vaults, however, requires further development of adaptable systems. In particular, the cargo capacity of capsids may be enlarged by devising multi-subunit cages, keeping in mind, however, an upper size limit owing to energetic cost, as shown for viral capsids [86]. Furthermore, the release of the cargo, such as RNA, from the capsid to the cytosol has to be increased, perhaps by shortening the packaging signal on the RNA, and splitting it into different elements, akin to AdVs, which contain multiple short stretches of A repeat (AT-rich) sequence elements at the left end of the genome ~~for directing the packaging process~~ [reviewed in 87]. These considerations highlight the importance of using combinatorial evolutionary selection processes in virus-like particle biogenesis, and considering the blueprints provided by viruses, such as adenovirus.

Conclusions

Viral nanomachines evolved by natural selection. They cycle between assembly in infected and disassembly in uninfected cells. Adenoviruses are among the best studied of them, and have contributed to a detailed understanding of the viral nature and the functioning of cells and organisms. Currently, the state-of-the-art in virus research extends from population averaged structural analyses and infection studies towards structural, physical and biological analyses of single virions and single infected cells,

for example by atomic force microscopy, nanofluidics and single cell genomics [88-90]. Detailed studies of the assembly and the disassembly processes with single virion resolution will continue to provide insights into the natural mechanisms of viruses including adenoviruses, and inspire the evolution of synthetic viruses for precision delivery in human and animal therapies.

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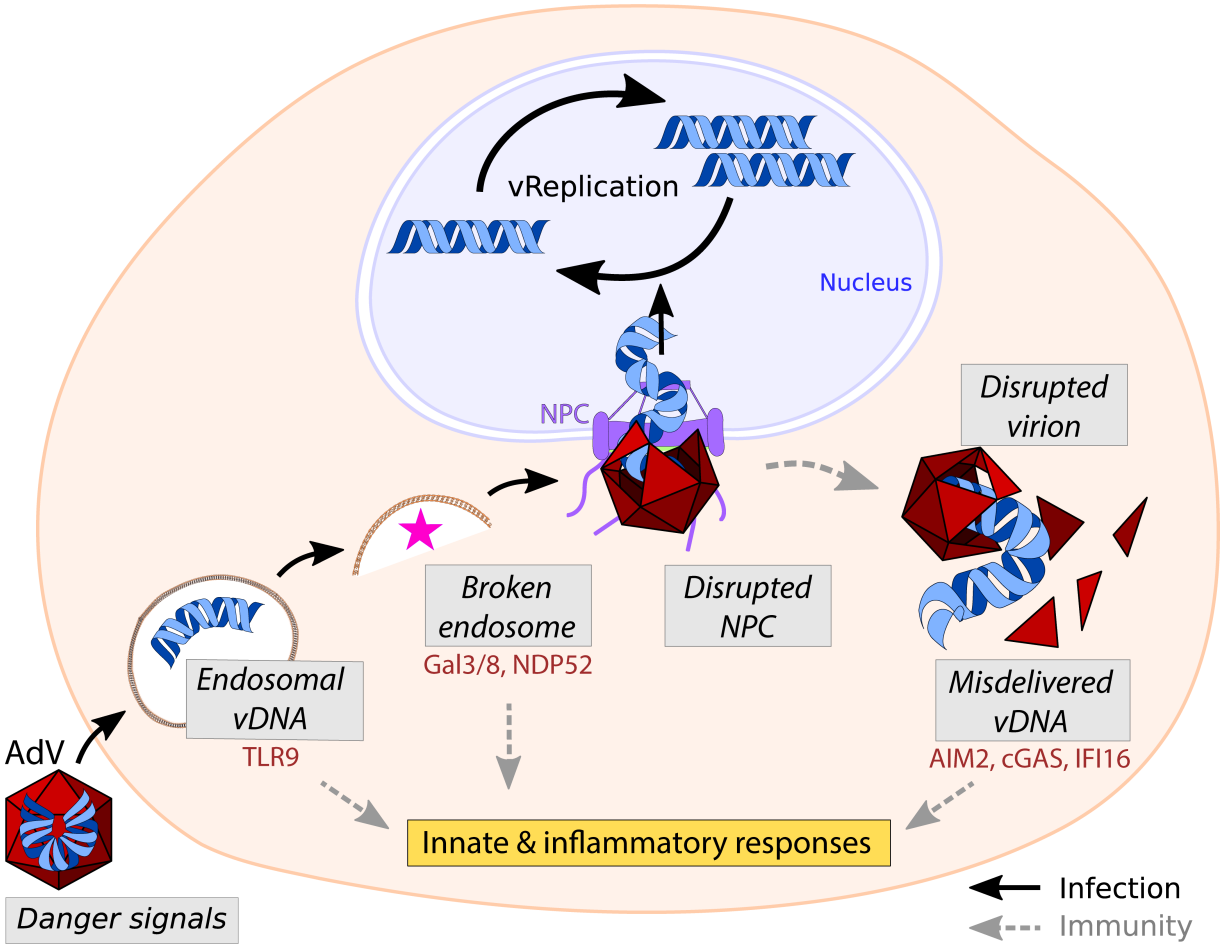
Fig. 1: Cost and effect of AdV entry into cells

Adenovirus entry into cells delivers danger signals activating pattern recognition receptors, such as toll-like receptor 9 (TLR9) recognizing double-stranded endosomal DNA, galectin 3 and 8 targeting broken membranes to autophagy through the adaptor NDP52, and sensors detecting abnormal DNA, including interferon-inducible protein absent in melanoma 2 (AIM2), cyclic GMP-AMP synthase (cGas) and interferon gamma inducible protein 16 (IFI16). Together, these activated sensors trigger cell type specific innate and inflammatory immune responses, which typically negatively regulate viral gene expression and replication in the cell nucleus. For additional information, please see main text and [29].



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Supplemental Figure

Human cervical epithelial HeLa cells grown on sapphire coverslips were infected with HAdV-C5 at a high multiplicity in presence of leptomycin B for 5 h, high pressure frozen using liquid nitrogen at 2100 bar, and freeze-substituted. Thin sections of 70 nm thickness were imaged using transmission electron microscopy at the Center for Microscopy and Image Analysis (ZMB) of the University of Zurich. Virus particle (V), microtubule (MT), mitochondrium (Mi). Pixel size 0.8 nm, scale bar 500 nm.